Plants having improved growth characteristics and a method for making the same

The present invention concerns a method for improving plant growth characteristics. More specifically, the present invention concerns a method for improving plant growth characteristics by increasing, in a plant, expression of a cell cycle switch gene encoding a 52kDa protein (CCS52 protein) and/or by increasing activity of the CCS52 protein itself. The present invention also concerns plants having increased expression of a nucleic acid encoding a CCS52 protein and/or increased activity of a CCS52 protein, which plants have improved growth characteristics relative to corresponding wild-type plants.

Given the ever-increasing world population, it remains a major goal of agricultural research to improve the efficiency of agriculture. Conventional means for crop and horticultural improvements utilise selective breeding techniques to identify plants having desirable characteristics. However, such selective breeding techniques have several drawbacks, namely that these techniques are typically labour intensive and result in plants that often contain heterogenous genetic complements that may not always result in the desirable trait being passed on from parent plants. In contrast, advances in molecular biology have allowed mankind to more precisely manipulate the germplasm of plants. Genetic engineering of plants entails the isolation and manipulation of genetic material (typically in the form of DNA or RNA) and the subsequent introduction of that genetic material into a plant. Such technology has led to the development of plants having various improved economic, agronomic or horticultural traits. A trait of particular economic interest is high yield.

The ability to improve one or more plant growth characteristics, would have many applications in areas such as crop enhancement, plant breeding, production of ornamental plants, arboriculture, horticulture, forestry, production of algae or plants (for use as bioreactors for example, for the production of pharmaceuticals, such as antibodies or vaccines, or for the bioconversion of organic waste, or for use as fuel, in the case of high-yielding algae and plants).

CCS52 belongs to a small group of proteins containing several WD repeat motifs and is the plant homologue of animal APC activators involved in mitotic cyclin degradation (WO99/64451). In Cebolla et al. (EMBO J., 1999, 18: 4476-84), the isolation of CCS52 clones from *Medicago sativa* root nodules was reported and CCS52 was described to be part of a small gene family that appears to be conserved in plants. Furthermore, the functional domains and regulation mechanisms of CCS52 proteins have been described in detail by Tarayre et al. (The plant Cell, 2004, vol 16, 422-434).

In document WO99/64451 it was suggested that downregulation of CCS52 expression pushes the cells towards proliferation and that overproduction of CCS52 pushes the cells towards differentiation. Also, in the document in the name of Kondorosi et al. (1999, The EMBO J. 18 (16), p.4476-4484), it is stated that expression of CCS52 may switch proliferating cells to differentiation programs. For some cells differentiation means endoreduplication. This switch to differentiation (or endoreduplication) clearly involves an arrest in proliferation, thus an arrest in cell division. These data were in line with earlier findings in yeast that teach when CCS52 is used to increase differentiation (or endoreduplication), a cell cycle arrest is inevitably triggered. Therefore, the effect on endoreduplication on the one hand, namely the increased cell size, is inherently linked to a reduction of cell number due to cell division arrest. The results obtained in *Medicago* and *Arabidopsis*, for CCS52 overexpression driven by the CaMV35S promoter corroborated this view.

The examples in document WO99/64451 show that *Medicago* plants expressing an anti-sense version of a *Medicago* CCS52 gene form fewer seeds and fewer lateral branches. Furthermore, constructs for overexpression of a *Medicago* CCS52 gene, under control of a strong constitutive promoter (CaMV35S), have been disclosed and were used to transform *Medicago* plants. Although it was indicated that overexpression of a CCS52 gene under the

control of a CaMV35S promoter resulted in a positive effect on somatic embryogenesis, no plants were regenerated and no further positive effects were observed. To the contrary, evidence has been presented that overexpression of CCS52 under the control of a CaMV35S promoter is detrimental. This detrimental effect was first observed in *Medicago* transgenic plants. Later, this detrimental effect was also observed in *Arabidopsis thaliana* transformed with the *Arabidopsis* CCS52 gene under control of a CaMV35S promoter.

Therefore, the prior art does not teach how the CCS52 gene can be used to improve plant growth characteristics, and so far only negative results with respect to the use of CCS52 for growth improvement have been obtained.

Unexpectedly, it has now been found that, in contrast to earlier observations, overexpression of a CCS52 gene does not cause a detrimental effect. Moreover, it has now been found that plant growth characteristics may even be improved by the methods of the present invention. These improved growth characteristics are obtained when overexpression of a CCS52 gene in a plant is controlled by an medium-strength promoter.

Further surprisingly, it has also been found that plants made by the methods of the present invention have specific characteristics such as increased plant size, increased organ size and/or increased number of organs, compared to corresponding wild-type plants.

Therefore, the present invention teaches how to improve plant growth characteristics, such as plant size, organ size and/or organ number by increased expression in a plant of a nucleic acid encoding a CCS52 protein.

According to a first embodiment of the present invention, there is provided a method to improve plant growth characteristics relative to corresponding wild-type plants, comprising the introduction into a plant of a

nucleic acid encoding a CCS52 protein, under control of a medium-strength promoter.

The introduction into a plant of a nucleic acid encoding a CCS52 protein under control of a medium-strength promoter, may result in an increased expression of the nucleic acid encoding a CCS52 protein. Additionally, this introduction may result in an increased level and/or activity of the CCS52 protein.

Advantageously, and according to a preferred embodiment of the present invention, increased expression of a nucleic acid encoding a CCS52 protein and/or increased level and/or activity of the CCS52 protein itself may be effected by a direct recombinant approach, for example, by transforming the plant with a nucleic acid encoding a CCS52 protein or a variant thereof.

Alternatively, increased expression of a nucleic acid encoding a CCS52 protein and/or increased level and/or activity of the CCS52 protein itself may be effected by an indirect recombinant approach, for example, by transforming a plant to modify the expression of a CCS52 gene already in that plant, which CCS52 gene may be endogenous or a transgene (previously) introduced into the plant. This may be effected by the inhibition or stimulation of regulatory sequences that drive expression of the endogenous gene or transgene. Such regulatory sequences may be introduced into a plant. For example, a medium-strength promoter may be introduced into a plant to drive the endogenous CCS52 gene, which medium-strength promoter may be heterologous to the endogenous CCS52 gene; Heterologous being not naturally occurring in the nucleic acid sequences flanking the CCS52 coding region when it is in its biological genomic environment.

The term "CCS52 protein" as used herein encompasses a cell cycle switch gene encoding a 52kDa protein and this term also encompasses variants thereof. Examples of CCS52 proteins are herein represented by SEQ

ID NO 2, 4 or 6. Other examples of CCS52 proteins are described in Cebolla et al. (EMBO 1999, vol. 18(16) 4476-4484) and in Tarayre et al. (The plant cell, 2004, vol. 16: 422-434). The terms "CCS52 nucleic acid" or "CCS52 gene" or "nucleic acid encoding a CCS52 protein" are used interchangeably herein and encompass, for example, nucleic acids as represented by SEQ ID NO 1, 3 or 5, or variants thereof. A variant CCS52 protein or a variant nucleic acid encoding a CCS52 protein include:

(i) Functional portions of a CCS52 nucleic acid, for example of SEQ ID NO 1, 3 or 5;

(ii) Nucleic acids capable of hybridising with a CCS52 nucleic acid, for example with SEQ ID NO 1, 3 or 5;

(iii) Alternative splice variants of a CCS52 nucleic acid, for example of SEQ ID NO 1, 3 or 5;

(iv) Allelic variants of a CCS52 nucleic acid, for example of SEQ ID NO 1, 3 or 5;

(v) Homologues of a CCS52 protein, for example of SEQ ID NO 2, 4 or 6;

(vi) Derivatives of a CCS52 protein, for example of SEQ ID NO 2, 4 or 6; and

(vii) Active fragments of a CCS52 protein, for example of SEQ ID NO 2, 4 or 6.

According to a preferred embodiment, such variants are (or encode) proteins having at least one of the conserved CCS52 motifs as described hereinafter.

According to a preferred embodiment, such variants are (or encode) proteins having CCS52 activity, or are (or encode) proteins that retain similar biological activity or at least part of the biological activity of a CCS52 protein. The biological activity of a CCS52 protein may be tested as described in Cebolla et al., 1999. This test involves overexpressing the CCS52 or variant in *Saccharomyces pombe*. The phenotypes of the transformed yeast cells are

compared with the phenotypes of yeast cells transformed with the empty vector pREP1 as negative control, and with the phenotypes of the yeast cells transformed with the pREP1-srw1$^+$ as positive control. Expression of either srw1$^+$ or CCS52 should result in growth arrest of the cells.

Advantageously, the methods according to the invention may be practised using variant CCS52 proteins and variant CCS52 nucleic acids. Suitable variants include variants of SEQ ID NO 2, 4 or 6 and/or variants of SEQ ID NO 1, 3 or 5.

The term "variant" includes variants in the form of a complement, DNA, RNA, cDNA or genomic DNA. The variant nucleic acid may be synthesized in whole or in part, it may be a double-stranded nucleic acid or a single-stranded nucleic acid. Also, the term "variant" encompasses a variant due to the degeneracy of the genetic code, a family member of the gene or protein and variants that are interrupted by one or more intervening sequences, such as introns, spacer sequences or transposons.

One variant nucleic acid encoding a CCS52 protein is a functional portion of a nucleic acid encoding a CCS52 protein. Advantageously, the method of the present invention may also be practised using a portion of a nucleic acid encoding a CCS52 protein. A functional portion refers to a piece of DNA derived from an original (larger) DNA molecule, which portion, retains at least part of the functionality of the original DNA, which functional portion, when expressed in a plant, gives plants having improved growth characteristics. The portion may be made by one or more deletions and/or truncations of the nucleic acid. Techniques for making such deletions and/or truncations are well known in the art. Portions suitable for use in the methods according to the invention may readily be determined by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the portion.

Another variant of a nucleic acid encoding a CCS52 protein is a nucleic acid capable of hybridising with a nucleic acid encoding a CCS52 protein, for example with any of the nucleic acids as represented by SEQ ID NO 1, 3 or 5. Hybridising sequences suitable for use in the methods according to the invention may readily be determined, for example by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the hybridising sequence.

The term "hybridising" as used herein means annealing to a substantially homologous complementary nucleotide sequences in a hybridization process. The hybridisation process may occur entirely in solution, i.e. both complementary nucleic acids are in solution. Tools in molecular biology relying on such a process include the polymerase chain reaction (PCR; and all methods based thereon), subtractive hybridisation, random primer extension, nuclease S1 mapping, primer extension, reverse transcription, cDNA synthesis, differential display of RNAs, and DNA sequence determination. The hybridisation process may also occur with one of the complementary nucleic acids immobilised to a matrix such as magnetic beads, Sepharose beads or any other resin. Tools in molecular biology relying on such a process include the isolation of poly (A+) mRNA. The hybridisation process may furthermore occur with one of the complementary nucleic acids immobilised to a solid support such as a nitro-cellulose or nylon membrane or immobilised by e.g. photolithography to e.g. a siliceous glass support (the latter known as nucleic acid arrays or microarrays or as nucleic acid chips). Tools in molecular biology relying on such a process include RNA and DNA gel blot analysis, colony hybridisation, plaque hybridisation, *in situ* hybridisation and microarray hybridisation. In order to allow hybridisation to occur, the nucleic acid molecules are generally thermally or chemically denatured to melt a double strand into two single strands and/or to remove hairpins or other secondary structures from single stranded nucleic acids. The stringency of hybridisation is influenced by conditions such as temperature, sodium/salt concentration and hybridisation buffer composition. High stringency conditions for hybridisation

include high temperature and/or low salt concentration (salts include NaCl and Na_3-citrate) and/or the inclusion of formamide in the hybridisation buffer and/or lowering the concentration of compounds such as SDS (sodium dodecyl sulphate detergent) in the hybridisation buffer and/or exclusion of compounds, such as dextran sulphate or polyethylene glycol (promoting molecular crowding) from the hybridisation buffer. Conventional hybridisation conditions are described in, for example, Sambrook (2001) Molecular Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York, but the skilled craftsman will appreciate that numerous different hybridisation conditions may be designed in function of the known or the expected sequence identity and/or length of the nucleic acids. Sufficiently low stringency hybridisation conditions are particularly preferred (at least in the first instance) to isolate nucleic acids heterologous to the DNA sequences of the invention defined supra. An example of low stringency conditions is 4-6x SSC / 0.1-0.5% w/v SDS at 37-45°C for 2-3 hours. Depending on the source and concentration of the nucleic acid involved in the hybridisation, alternative conditions of stringency may be employed, such as medium stringency conditions. Examples of medium stringency conditions include 1-4x SSC / 0.25% w/v SDS at ≥ 45°C for 2-3 hours. Preferably, the variants capable of hybridizing with a CCS52 gene are capable of specifically hybridizing. With "specifically hybridizing" is meant hybridising under stringent conditions. An example of high stringency conditions includes 0.1-2XSSC, 0.1XSDS, and 1X SSC, 0.1X SDS at 60°C for 2-3 hours.

The methods according to the present invention may also be practised using an alternative splice variant of a nucleic acid encoding a CCS52 protein, for example, an alternative splice variant of SEQ ID NO 1, 3 or 5. The term "alternative splice variant" as used herein encompasses variants of a nucleic acid in which selected introns and/or exons have been excised, replaced or added. Such splice variants may be found in nature or may be manmade. Methods for making such splice variants are well known in the art. Splice variants suitable for use in the methods according to the invention may readily

be determined, for example, by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the splice variant.

Another variant CCS52 nucleic acid useful in practising the method for improving plant growth characteristics, is an allelic variant of a CCS52 gene, for example, an allelic variant of SEQ ID NO 1, 3 or 5. Allelic variants exist in nature and encompassed within the methods of the present invention is the use of these natural alleles. Allelic variants also encompass Single Nucleotide Polymorphisms (SNPs) as well as Small Insertion/Deletion Polymorphisms (INDELs). The size of INDELs is usually less than 100 bp. SNPs and INDELs form the largest set of sequence variants in naturally occurring polymorphic strains of most organisms. Allelic variants suitable for use in the methods according to the invention may readily be determined, for example, by following the methods described in the Examples section by simply substituting the sequence used in the actual Example with the allelic variant.

The present invention provides a method for improving plant growth characteristics, comprising increasing expression in a plant of an alternative splice variant or of an allelic variant of a nucleic acid encoding a CCS52 protein and/or by increasing the level and/or activity in a plant of a CCS52 protein encoded by an alternative splice variant or allelic variant.

One example of a variant CCS52 protein useful in practising the methods of the present invention is a homologue of a CCS52 protein. "Homologues" of a CCS52 protein encompass peptides, oligopeptides, polypeptides, proteins and enzymes having an amino acid substitution, deletion and/or insertion relative to the CCS52 protein in question and having similar biological and functional activity as the CCS52. Homologues of a CCS52 protein may be manmade via the techniques of genetic engineering and/or protein engineering. To produce such homologues, amino acids of the protein may be replaced by other amino acids having similar properties (such as

similar hydrophobicity, hydrophilicity, antigenicity, propensity to form or break α-helical structures or β-sheet structures). Conservative substitution tables are well known in the art (see for example Creighton (1984) Proteins. W.H. Freeman and Company).

Homologues of a particular CCS52 protein may exist in nature and may be found in the same or different species or organism from which the particular CCS52 protein is derived. Two special forms of homologues, orthologues and paralogues, are evolutionary concepts used to describe ancestral relationships of genes. The term "orthologues" relates to genes in different organisms that are homologous due to ancestral relationship. The term "paralogues" relates to gene-duplications within the genome of a species leading to paralogous genes. The term "homologues" as used herein also encompasses paralogues and orthologues of a CCS52 protein, which are also useful in practising the methods of the present invention.

Another special form of a CCS52 homologue is a member of the same gene family of CCS52 proteins. It is known that AtCCS52A1 belongs to a multigene family, and therefore a person skilled in the art will recognize that the methods according to the present invention may also be practised using the encoding sequence of a family member of a CCS52 protein, such as a family member of SEQ ID NO 2, 4 or 6.

The homologues useful in the method according to the invention have in increasing order of preference, at least 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a CCS52 protein, for example, to any one of SEQ ID NO 2, 4 or 6. Alternatively, the nucleic acid sequence encoding any one of the above-mentioned homologue may have at least 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%,

66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity to a CCS52 nucleic acid, for example, to any one of SEQ ID NO 1, 3 or 5.

The percentage of sequence identity as mentioned above, between proteins or nucleic acids, may be calculated using a pairwise global alignment program implementing the algorithm of Needleman-Wunsch (J. Mol. Biol. 48: 443-453, 1970), which maximizes the number of matches and keeps the number of gaps to a minimum. For calculation of the above-mentioned percentages, the program needle (EMBOSS package) may be used with a gap opening penalty of 10 and gap extension penalty of 0.1. For proteins, the blosum62 matrix with a word length of 3 is preferably used. For nucleic acids, the program needle uses the matrix "DNA-full", with a word-length of 11, as provided by the EMBOSS package. The Needleman-Wunsch algorithm is best suited for analysing related protein sequences over their full length.

The homologues useful in the methods according to the invention (the proteins or their encoding nucleic acid sequences) may be derived (either directly or indirectly (if subsequently modified) from any source as described hereinafter, provided that the sequence, when expressed in a plant, leads to improved plant growth characteristics. The nucleic acid (or protein) may be isolated from yeast, fungi, plants, algae, insects or animals (including humans). This nucleic acid may be substantially modified from its native form in composition and/or genomic environment through deliberate human manipulation.

The nucleic acid encoding a CCS52 homologue is preferably isolated from a plant. Examples of CCS52 proteins are *Arabidopsis thaliana* CCS52A1 (SEQ ID NO 2 and corresponding encoding sequence SEQ ID NO 1), *Oryza sativa* CCS52A (SEQ ID NO 4 and corresponding encoding sequence SEQ ID

NO 3), and *Oryza sativa* CCS52B (SEQ ID NO 6 and corresponding genomic sequence SEQ ID NO 5).

CCS52 proteins of *Arabidopsis thaliana and Medicago sativa* have been subdivided into different classes (Cebolla et al., 1999, EMBO J. 18: p 4476-4484). Class CCS52A (with A1 and A2 isoforms) and class CCS52B (with the B1 isoform). These classes and isoforms are also encompassed by the term "homologue" as used herein. Advantageously, these different classes and isoforms of CCS52 proteins, or their encoding nucleic acids, may be used in the methods of the present invention. Accordingly, the present invention provides a method as described hereinabove, wherein the CCS52 nucleic acid or CCS52 protein is obtained from a plant, preferably from a dicotyledoneous plant, further preferably from the family *Brassicaceae*, more preferably from *Arabidopsis thaliana*. According to a further embodiment, CCS52 is CCS52A or CCS52B. According to a further embodiment of the invention, CCS52 is a CCS52A1 protein. A person skilled in the art will recognize that a "CCS52A1" is a protein being closer related to AtCCS52A1, than to AtCCS52A2 or AtCCS52B. This closer relationship may be determined by calculating percentage of sequence identity, or by comparing the presence of conserved motifs as described hereinafter.

Still other suitable CCS52 homologues and their encoding sequences may be found in (public) sequence databases. Methods for the search and identification of CCS52 protein homologues in sequence databases would be well within the realm of a person skilled in the art. Such methods, involve screening sequence databases with the sequences provided by the present invention, for example, SEQ ID NO 2, 4 or 6 (or SEQ ID NO 1, 3 or 5), preferably in a computer readable form. Useful sequence databases include, but are not limited, to Genbank (http://www.ncbi.nlm.nih.gov/web/Genbank), the European Molecular Biology Laboratory Nucleic acid Database (EMBL) (http:/w.ebi.ac.uk/ebi-docs/embl-db.html) or versions thereof or the MIPS database (http://mips.gsf.de/). Different search algorithms and software for the

alignment and comparison of sequences are well known in the art. Such software includes for example, GAP, BESTFIT, BLAST, FASTA and TFASTA. Preferably the BLAST software is used, which calculates percent sequence identity and performs a statistical analysis of the similarity between the sequences. The suite of programs referred to as BLAST programs has 5 different implementations: three designed for nucleotide sequence queries (BLASTN, BLASTX, and TBLASTX) and two designed for protein sequence queries (BLASTP and TBLASTN) (Coulson, Trends in Biotechnology: 76-80, 1994; Birren et al., GenomeAnalysis, 1: 543, 1997). The software for performing BLAST analysis is publicly available through the National Centre for Biotechnology Information.

Orthologues of a CCS52 protein in other plant species may easily be found by performing a reciprocal Blast search. This method comprises searching one or more sequence databases with a query gene or protein (for example, any one of SEQ ID NO 1 to 6), using for example, the BLAST program. The highest-ranking subject genes that result from this search are then used as a query sequence in a similar BLAST search. Only those genes that have as a highest match again the original query sequence are considered to be orthologous genes. For example, to find a rice orthologue of an *Arabidopsis thaliana* gene, one may perform a BLASTN or TBLASTX analysis on a rice database such as the *Oryza sativa Nipponbare* database available at the NCBI website (http://www.ncbi.nlm.nih.gov). In a next step, the highest ranking rice sequences are used in a reverse BLAST search on an *Arabidopsis thaliana* sequence database. The method may be used to identify orthologues from many different species, for example, from corn.

Paralogues of a CCS52 protein in the same species may easily be found by performing a Blast search on sequences of the same species from which the CCS52 protein is derived. From the sequences that are selected by the Blast search, the true paralogues may be identified by looking for the highest

sequence identity or for the highest conservation of typical CCS52 motifs as described hereinafter.

Homologues of a AtCCS52A1 protein, as represented by SEQ ID NO 2, and their encoding sequences, may be found in many different species. Examples of such homologues are presented in the phylogenetic tree in Figure 12. The homologues are presented by their Genbank accession number. Preferred homologues to be used in the present invention are the homologues that group close to AtCCS52A1_At4g22910, for example, those homologues that group between OsAP003298.3 and Hs19_NP_057347.1. These homologues include but are not limited to Hs19_NP_057347.1, Mm_NP_062731, XL-CAA74576.1, Ggcdh1c_AAL31949, Ggcdh1b_AAL31948.1, Ggcdh1d_AAL31950, Ggcdh1a_AAL31947, Dm_NP_726941, Ag_agCP12792, Ce_NP_496075.1, Dm_NP_611854, and the homologues grouping closest to AtCCS52A1_At4g22910, including Le_AW0030735, AtCCS52A2_At4g11920, MtCCS52A_AF134835, Gm_BG044933, Os_AK070642, Zm_AY112458, AtCCS52B_At5g13840, MsCCSB, Gm_AI736659 and Zm_AI861254. The genome sequences of *Arabidopsis thaliana* and *Oryza sativa* are now available in public databases such as Genbank and other genomes are currently being sequenced. Therefore, it is expected that further homologues will readily be identifiable by sequence alignment with any one of SEQ ID NO 1 to 6 using the programs BLASTX or BLASTP or other programs.

The above-mentioned software analyses for comparing sequences, for the calculation of sequence identity, for the search of homologues, orthologues or paralogues or for the making of a phylogenetic tree, is preferentially done with full-length sequences. Alternatively, these software analyses may be carried out with a conserved region of the CCS52 protein or nucleic acid sequence, as described hereinafter. Accordingly, these analyses may be based on the comparison and calculation of sequence identity between conserved regions, functional domains, motifs or boxes.

The identification of protein domains, motifs and boxes, would also be well within the realm of a person skilled in the art by using protein domain information as available in the PRODOM (http://www.biochem.ucl.ac.uk/bsm/dbbrowser/jj/prodomsrchjj.html), PIR (http://pir.georgetown.edu/), PROSITE (http://au.expasy.org/PROSITE/) or pFAM (http://pFAM.wustl.edu/) databases. Software programs designed for such domain searching include, but are not limited to, MotifScan, MEME, SIGNALSCAN, and GENESCAN. MotifScan is a preferred software program and is available at (http://hits.isb-sib.ch/cgi-bin/PFSCAN, which program uses the protein domain information of PROSITE and pFAM . A MEME algorithm (Version 3.0) may be found in the GCG package; or at http://www.sdsc.edu/MEME/meme. SIGNALSCAN version 4.0 information is available at http://biosci.cbs.umn.edu/software/sigscan.html. GENESCAN may be found at http://gnomic.stanford.edu/GENESCANW.html.

Ten conserved motifs have been identified in CCS52 proteins and the consensus sequences for these motifs are represented herein by SEQ ID NO 7 to 16 (see Figure 13). Preferably, these motifs are used to search databases and to identify homologous CCS52 sequences. The presence of these motifs (for example, as represented by SEQ ID NO 7 to 16), may be determined by screening proteins sequences for sequence identity with these consensus motifs. Another aspect of the present invention is the use of conserved CCS52 motifs as represented by ant one of SEQ ID NO 7 to 15, to identify, or to manufacture (via protein engineering or grafting of such motifs into a target protein), homologues of a CCS52 gene or protein which are capable of improving plant growth characteristics. The N-terminal conserved motif, the C-box (SEQ ID NO 16) is further described in Tarayre et al. 2004.

Preferred CCS52 homologues useful in the methods of the present invention are plant CCS52 proteins that comprise at least 4 of the aforementioned consensus motifs. Motif number 2, as represented by SEQ ID

NO 8 has also been described as a N-terminal "CSM" motif in Tarayre et al., 2004. Motif number 9, as represented by SEQ ID NO 15, is presumably involved in the interaction with other proteins; it is a C-terminal IR motif, which has been described as necessary for the functionality of CCS52 in the APC complex. Furthermore, the presence of multiple conserved motifs (SEQ ID NO 7 to 16) strongly suggests that CCS52 proteins are involved in multiple interactions and that several CCS52 target genes/proteins exist. Further details on the relationship between the IR motif and the CCS52 functionality are described in Tarayre et al. (2004, Plant Cell.,16(2): 422-34), which document is herein incorporated by reference as if fully set forth.

Figure 13 shows the individual conserved motifs of different CCS52 proteins as well as the consensus sequences thereof, which are herein represented by SEQ ID NO 7 to 16. A person skilled in the art will recognize that a CCS52 motif may deviate, by for example 1 or 2 mismatches, from the abovementioned consensus CCS52 motifs, without losing its functionality. One example of such a deviation is number of "X" amino acids in motif 3.

As may be deducted from Figure 13, the consensus sequences may be more defined when only taking CCS52A proteins into account. For example, for CCS52A proteins, Motif number 1 has G on position 1, N at position 3, F or L at position 4, A at position 5, L at position 6 and L or I at position 9. This consensus Motif 1 for CCS52A proteins is represented herein by SEQ ID NO 17. For CCS52A proteins, Motif number 7 has T at position 5 and H at position 8. Also, for CCS52A proteins, Motif number 9 has "I" at position 2 and "R" at position 9.

Some of the variants as mentioned hereinabove may occur in nature and may be isolated from nature. Once the sequence of a variant is known, and its corresponding encoding sequence, the person skilled in the art will be able to isolate the corresponding CCS52 gene or variant from biological material such as genomic libraries, for example, by the technique of PCR. One example

of such an experiment is outlined in Example 1. Alternatively, when the exact sequence is not known, new CCS52 proteins may be isolated from biological material via hybridization techniques based on probes from known CCS52 proteins.

Alternatively and/or additionally, some variants as mentioned above may be manmade via techniques involving, for example, mutation (substitution, insertion or deletion) or derivation. These variants are herein referred to as "derivatives", which derivatives are also useful in the methods of the present invention. Derivatives of a protein may readily be made using peptide synthesis techniques well known in the art, such as solid phase peptide synthesis and the like, or by protein engineering via recombinant DNA manipulations. The manipulation of DNA sequences to produce substitution, insertion or deletion variants of a protein are well known in the art. For example, techniques for making substitution mutations at predetermined sites in DNA are well known to those skilled in the art and include M13 mutagenesis, T7-Gen in vitro mutagenesis (USB, Cleveland, OH), QuickChange Site Directed mutagenesis (Stratagene, San Diego, CA), PCR-mediated site-directed mutagenesis or other site-directed mutagenesis protocols.

One example of a derivative is a substitutional variant. The term "substitutional variants" of a CCS52 protein refers to those variants in which at least one residue in an amino acid sequence has been removed and a different amino acid inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions usually are of the order of about 1-10 amino acids, and deletions can range from about 1-20 amino acids. Preferably, amino acid substitutions comprise conservative amino acid substitutions.

Other derivatives are "insertional variants" in which one or more amino acids are introduced into a predetermined site in the CCS52 protein. Insertions may comprise amino-terminal and/or carboxy-terminal fusion as well as intra-

sequence insertion of single or multiple amino acids. Generally, insertions within the amino acid sequence are of the order of about 1 to 10 amino acids. Examples of amino- or carboxy-terminal fusions include fusion of the binding domain or activation domain of a transcriptional activator as used in the yeast two-hybrid system, phage coat proteins, (histidine)6-tag, glutathione S-transferase-tag, protein A, maltose-binding protein, dihydrofolate reductase, Tag·100 epitope, c-myc epitope, FLAGâ-epitope, lacZ, CMP (calmodulin-binding peptide), HA epitope, protein C epitope and VSV epitope.

Other derivatives of a CCS52 protein are "deletion variants", characterised by the removal of one or more amino acids from the protein.

Another derivative of a CCS52 protein is characterised by substitutions, and/or deletions and/or additions of naturally and non-naturally occurring amino acids compared to the amino acids of a naturally-occurring CCS52 protein. A derivative may also comprise one or more non-amino acid substituents compared to the amino acid sequence from which it is derived. Such non-amino acid substituents include for example, non-naturally occurring amino acids, a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence. Such a reporter molecule may be bound to facilitate the detection of the CCS52 protein.

Another variant of a CCS52 protein useful in the methods of the present invention is an active fragment of a CCS52 protein. "Active fragments" of a CCS52 protein encompass at least five contiguous amino acid residues of a CCS52 protein, which residues retain similar biological and/or functional activity to a naturally occurring protein or a part thereof. Suitable fragments include fragments of a CCS52 protein starting at the second or third or further internal methionine residues. These fragments originate from protein translation, starting at internal ATG codons, whilst retaining its functionality in the methods of the present invention. Suitable functional fragments of a CCS52 protein, or suitable portions of nucleic acids that correspond to such fragments, useful in

the methods of the present invention, may have one or more of the conserved motifs of CCS52 proteins as represented by SEQ ID NO 7 to 16, whilst retaining its functionality in the methods of the present invention. One particular example of a functional fragment is a fragment of a rice CCS52 protein, for example of SEQ ID NO 6, which ends with the IR motif.

According to a preferred embodiment of the present invention, a method to improve plant growth characteristics comprises increased expression of a nucleic acid encoding a CCS52 protein. Methods for obtaining increased expression of genes or gene products (proteins) are well documented in the art and include, for example, overexpression driven by an operably linked promoter, or the use of transcription enhancers or translation enhancers. The term overexpression as used herein means any form of expression that is additional to the original wild-type expression level. Preferably the nucleic acid to be introduced into the plant and/or the nucleic acid that is to be overexpressed in the plant is in the sense direction with respect to the promoter to which it is operably linked. Preferably, in the methods of the present invention a nucleic acid encoding a CCS52 protein is overexpressed in a plant, such as a CCS52 nucleic acid of SEQ ID NO 1.

Alternatively and/or additionally, increased expression of a CCS52 gene or increased level, and/or activity of a CCS52 protein in a plant cell, may be achieved by mutagenesis. For example, the mutations may be responsible for altered control of an endogenous CCS52 gene, resulting in more expression of the gene, relative to the wild-type gene. Mutations can also cause conformational changes in a protein, resulting in higher levels and/or more activity of the CCS52 protein. Such mutations or such mutant genes may be selected, or isolated and/or introduced into the same or different plant species in order to obtain plants having improved growth characteristics. Examples of such mutants include dominant positive mutants of a CCS52 gene.

According to a further aspect of the present invention, there is provided genetic constructs and vectors to facilitate introduction and/or to facilitate expression and/or to facilitate maintenance in a host cell of the nucleic acids useful in the methods according to the invention. Therefore, according to a further embodiment of the present invention, there is provided a genetic construct comprising:

(a) a nucleic acid encoding a CCS52 protein or a variant thereof; operably linked to

(b) a medium-strength promoter; and optionally

(c) a transcription termination sequence.

Constructs useful in the methods according to the present invention may be constructed using recombinant DNA technology well known to persons skilled in the art. The gene constructs may be inserted into vectors, which may be commercially available, suitable for transforming into plants and suitable for maintenance and expression of the gene of interest in the transformed cells. Preferably, the genetic construct according to the present invention is a plant expression vector, suitable for introduction and/or maintenance and/or expression of a nucleic acid in a plant cell, tissue, organ or whole plant.

The nucleic acid according to (a) is advantageously any of the nucleic acids described hereinbefore. A preferred nucleic acid is a nucleic acid represented by SEQ ID NO 1, 3 or 5, or a variant thereof as hereinbefore defined, or is a nucleic acid encoding a protein as represented by SEQ ID NO 2, 4 or 6, or a variant thereof as hereinbefore defined.

With the term "promoter" it meant a transcription control sequence. The promoter of (b) is operable in a plant, most preferably the promoter is derived from a plant sequence.

The terms "transcription control sequence" or "promoter" are used interchangeably herein and are to be taken in a broad context to refer to regulatory nucleic acids capable of effecting expression of the sequences to

which they are operably linked. Encompassed by the aforementioned terms are transcriptional regulatory sequences derived from a classical eukaryotic genomic gene (including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence) and additional regulatory elements (i.e. upstream activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner. Also included within the term is a transcriptional regulatory sequence of a classical prokaryotic gene, in which case it may include a –35 box sequence and/or –10 box transcriptional regulatory sequences. The term "regulatory element" also encompasses a synthetic fusion molecule or derivative, which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

The term "operably linked" as used herein refers to a functional linkage between the promoter sequence and the gene of interest, such that the promoter sequence is able to initiate transcription of the gene of interest. Preferably, the gene of interest is operably linked in the sense orientation to the promoter.

The term "medium-strength promoter" means a promoter other than a strong promoter and refers to the expression level in green vegetative tissues.

Advantageously, any promoter may be used for the methods of the invention, provided that it has a medium-strength expression pattern in green vegetative tissues. These promoters have, when compared to a strong constitutive promoter (such as the strong constitutive/ubiquitous CaMV35S promoter), a lower expression level at least in green vegetative tissues. Promoters useful in the methods of the present invention do not reach the same strong expression level in green vegetative tissue of a plant as the CaMV35S promoter.

Preferably, the medium-strength promoter is of overall medium-strength during vegetative growth of the plant. One example of such a promoter is the sunflower ubiquitin promoter.

The term "medium-strength promoter" clearly does not include a CaMV35S promoter, which is known to be a very strong promoter. To the contrary, a medium-strength promoter has an expression level in green vegetative tissue that is at least 10-fold lower than the CaMV35S promoter. A person skilled in the art will recognize that for many plant species the CaMV35S promoter activity has been measured and that in many different plant species, such as rice and corn, the level of activity of the CaMV35S promoter is very high.

One method to measure the promoter strength is through the use of promoter-beta-glucuronidase fusions. The promoter if hereby fused to the *Escherichia coli* uidA gene encoding beta-glucuronidase and the chimeric construct is transformed into a plant. Proteins are extracted from the plant material and GUS activity is measured (Jefferson et al., 1987, EMBO J. 20;6(13):3901-7). Promoter activity is then calculated as the optical density in units per mg of extracted protein.

Examples of measurements of CaMV35S expression levels have been described previously, for example for rice (Battraw and Hall, 1990, Plant Mol Biol. 15(4): 527-38), for tobacco (Jefferson et al. ,1987, EMBO J., 20-6(13): 3901-7) and for *Arabidopisis* (S. Planchais, PhD. thesis University of Ghent, 2000).

In the context of this invention, GUS activity is measured from vegetative tissues after germination. Preferably, these measurements are performed during vegetative growth of the plant, for example after 2, preferably after 4 weeks post germination.

According to one embodiment of the present invention, the medium-strength promoter is a constitutive promoter. The term "constitutive" as defined herein refers to a promoter that is expressed substantially continuously and substantially in all tissues of a plant. Examples of useful constitutive promoters are ubiquitin promoters (in case of monocots intron-less ubiquitin promoters), such as rice or maize ubiquitin promoters.

According to one particular embodiment of invention, the medium-strength promoter is the sunflower ubiquitin promoter (without intron). The term "medium-strength promoter" as used herein therefore also means a promoter that has the same or similar activity, as the sunflower ubiquitin promoter in *Arabidopsis thaliana*. Similar activity in this context means an activity that is at most 20-fold higher or lower than the sunflower ubiquitin promoter, preferably at most 10-fold higher or lower or 5-fold higher or lower or 3-fold higher or lower.

Alternatively and according to another embodiment of the invention, the medium-strength promoter is a tissue-preferred promoter, characterized by the fact that it shows medium-strength expression in green vegetative tissue. The term "tissue-specific" promoter is used interchangeably herein with a "tissue-preferred" promoter. A promoter useful in the methods of the present invention may have a strong expression level, in other parts of the plant but the green vegetative tissue. For example, the *Arabidopsis thaliana* 2S2 promoter, which confers strong expression in seeds, may be used for the methods of the present invention. Besides the 2S2 promoter, other suitable tissue-preferred promoters include pPROLAMIN or pOLEOSIN, or promoters that show strong expression in aleurone, embryo, scutellum or endosperm. One example of a useful young-tissue preferred promoter is the beta-expansin promoter.

In document WO99/64451, it was suggested to clone a CCS52 gene under control of the *endod12Ams* promoter or the *Srglb3* promoter in order to have a positive effect on differentiation and somatic embryogenesis. These

positive effects have never been shown. These promoters are disclaimed from the constructs of the present invention.

Optionally, in the genetic construct according to the invention, one or more terminator sequences may also be incorporated. The term "transcription termination sequence" encompasses a control sequence at the end of a transcriptional unit, which signals 3' processing and polyadenylation of a primary transcript and termination of transcription. Additional regulatory elements, such as transcriptional or translational enhancers, may be incorporated in the genetic construct. Those skilled in the art will be aware of terminator and enhancer sequences, which may be suitable for use in performing the invention. Such sequences would be known or may readily be obtained by a person skilled in the art.

The genetic constructs of the invention may further include an origin of replication, which is required for maintenance and/or replication in a specific cell type. One example is when a genetic construct is required to be maintained in a bacterial cell as an episomal genetic element (e.g. plasmid or cosmid). Preferred origins of replication include, but are not limited to, the f1-ori and colE1.

The genetic construct may optionally comprise a selectable marker gene. As used herein, the term "selectable marker gene" includes any gene, which confers a phenotype on a cell in which it is expressed to facilitate the identification and/or selection of cells, which are transfected or transformed with a genetic construct of the invention. Suitable markers may be selected from markers that confer antibiotic or herbicide resistance. Cells containing the recombinant DNA will thus be able to survive in the presence of antibiotic or herbicide concentrations that kill untransformed cells. Examples of selectable marker genes include genes conferring resistance to antibiotics (such as nptII encoding neomycin phosphotransferase capable of phosphorylating neomycin and kanamycin, or hpt encoding hygromycin phosphotransferase capable of

phosphorylating hygromycin), to herbicides (for example, bar which provides resistance to Basta; aroA or gox providing resistance against glyphosate), or genes that provide a metabolic trait (such as manA that allows plants to use mannose as sole carbon source). Visual marker genes result in the formation of colour (for example, beta-glucuronidase, GUS), luminescence (such as luciferase) or fluorescence (Green Fluorescent Protein, GFP, and derivatives thereof). Further examples of suitable selectable marker genes include the ampicillin resistance gene (Ampr), tetracycline resistance gene (Tcr), bacterial kanamycin resistance gene (Kanr), phosphinothricin resistance gene, and the chloramphenicol acetyltransferase (CAT) gene, amongst others

According to a further embodiment of the present invention, there is provided a method for the production of transgenic plants having improved growth characteristics relative to corresponding wild-type plants, comprising:

(a) introducing into a plant cell a CCS52 nucleic acid or a variant thereof, preferably introducing a genetic construct as described hereinabove;

(b) cultivating said plant cell under conditions promoting plant growth.

"Introducing" the CCS52 nucleic acid or the genetic construct into the plant cell is preferably achieved by transformation. The term "transformation" as used herein encompasses the transfer of an exogenous polynucleotide into a host cell, irrespective of the method used for transfer. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention. The choice of tissue depends on the particular plant species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem). The polynucleotide may be transiently or stably introduced into a host cell and may be maintained non-integrated, for example, as a plasmid. Alternatively, it may be integrated into the host genome. Preferably, the CCS52 nucleic acid is

stably integrated in the genome of the plant cell, which may be achieved, for example, by using a plant transformation vector or a plant expression vector having T-DNA borders, which flank the nucleic acid to be introduced into the genome.

Transformation of a plant species is now a fairly routine technique. Advantageously, any of several transformation methods may be used to introduce the gene of interest into a suitable ancestor cell. Transformation methods include the use of liposomes, electroporation, chemicals that increase free DNA uptake, injection of the DNA directly into the plant, particle gun bombardment, transformation using viruses or pollen and microprojection. Methods may be selected from the calcium/polyethylene glycol method for protoplasts (Krens, F.A. *et al.*, 1882, Nature 296, 72-74; Negrutiu I. *et al.*, June 1987, Plant Mol. Biol. 8, 363-373); electroporation of protoplasts (Shillito R.D. *et al.*, 1985 Bio/Technol 3, 1099-1102); microinjection into plant material (Crossway A. *et al.*, 1986, Mol. Gen Genet 202, 179-185); DNA or RNA-coated particle bombardment (Klein T.M. *et al.*, 1987, Nature 327, 70) infection with (non-integrative) viruses and the like. A preferred method for the production of transgenic plants according to the invention, is an *Agrobactierium*-mediated transformation method.

Transgenic rice plants are preferably produced via *Agrobacterium*-mediated transformation using any of the well-known methods for rice transformation, such as the ones described in any of the following: published European patent application EP1198985, Aldemita and Hodges (Planta, 1996, 199: 612-617,); Chan et al. (Plant Mol. Biol., 1993, 22 (3): 491-506,); Hiei et al. (Plant J., 1994, 6 (2): 271-282,); which disclosures are incorporated by reference herein as if fully set forth. In the case of corn transformation, the preferred method is as described in either Ishida et al. (Nat. Biotechnol., 1996, 14(6): 745-50) or Frame et al. (Plant Physiol., 2002, 129(1): 13-22), which disclosures are incorporated by reference herein as if fully set forth.

Generally after transformation, plant cells or cell groupings are selected for the presence of one or more markers, which are co-transformed with the CCS52 gene.

The resulting transformed plant cell, cell grouping, or plant tissue, may then be used to regenerate a whole transformed plant via regeneration techniques well known to persons skilled in the art. Therefore, cultivating the plant cell under conditions promoting plant growth, may encompass the steps of selecting and/or regenerating and/or growing to reach maturity.

Following DNA transfer and regeneration, putatively transformed plants may be evaluated, for instance using Southern analysis, for the presence of the gene of interest, copy number and/or genomic organisation. Alternatively or additionally, expression levels of the newly introduced DNA may be monitored using Northern and/or Western analysis, both techniques being well known to persons having ordinary skill in the art.

The generated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second generation (or T2) transformants, and the T2 plants further propagated through classical breeding techniques.

The generated transformed organisms may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed rootstock grafted to an untransformed scion).

The invention also includes host cells containing an isolated nucleic acid molecule encoding a CCS52 or a genetic construct as mentioned hereinbefore. Preferred host cells according to the invention are plant cells. Accordingly,

there is provided plant cells, tissues, organs and whole plants that have been transformed with a genetic construct of the invention.

The present invention clearly extends to plants obtainable by any of the methods as described hereinbefore, which plants have improved growth characteristics relative to corresponding wild-type plants. The present invention extends to plants, which have increased expression levels of a nucleic acid encoding a CCS52 protein and/or increased level and/or activity od a CCS52 protein. The present invention extends to plants containing a genetic construct as described hereinabove, which plants have improved growth characteristics.

The present invention clearly also extends to any plant cell or plant produced by any of the methods described herein, and to all plant parts and propagules thereof. The present invention extends further to encompass the progeny of a primary transformed cell, tissue, organ or whole plant that has been produced by any of the aforementioned methods, the only requirement being that progeny exhibit the same genotypic and/or phenotypic characteristic(s) as those produced in the parent by the methods according to the invention.

The invention also extends to any part of the plant according to the invention, preferably a harvestable part of a plant, such as, but not limited to, a seed, leaf, fruit, flower, stem culture, stem, rhizome, root, tuber, bulb and cotton fiber.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), and plant cells, tissues and organs. The term "plant" also therefore encompasses suspension cultures, embryos, meristematic regions, callus tissue, leaves, seeds, roots, shoots, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily *Viridiplantae*, in particular monocotyledonous and dicotyledonous plants including a fodder or

forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising *Acacia spp., Acer spp., Actinidia spp.,Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp, Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chaenomeles spp.,Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Diheteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehrartia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia villosa, Fagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp, Freycinetia banksii, Geranium thunbergii, Ginkgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp., Hemarthia altissima, Heteropogon contortus, Hordeum vulgare, Hyparrhenia rufa, Hypericum erectum, Hyperthelia dissoluta, Indigo incarnata, Iris spp., Leptarrhena pyrolifolia, Lespediza spp., Lettuca spp., Leucaena leucocephala, Loudetia simplex, Lotonus bainesii, Lotus spp., Macrotyloma axillare, Malus spp., Manihot esculenta, Medicago sativa, Metasequoia glyptostroboides, Musa sapientum, Nicotianum spp., Onobrychis spp., Ornithopus spp., Oryza spp., Peltophorum africanum, Pennisetum spp., Persea gratissima, Petunia spp., Phaseolus spp., Phoenix canariensis, Phormium cookianum, Photinia spp., Picea glauca, Pinus spp., Pisum sativum, Podocarpus totara, Pogonarthria fleckii, Pogonarthria squarrosa, Populus spp., Prosopis cineraria, Pseudotsuga menziesii, Pterolobium stellatum, Pyrus communis, Quercus spp., Rhaphiolepsis umbellata, Rhopalostylis sapida, Rhus natalensis, Ribes grossularia, Ribes spp., Robinia pseudoacacia, Rosa spp., Rubus spp., Salix spp., Schyzachyrium sanguineum, Sciadopitys verticillata, Sequoia sempervirens, Sequoiadendron giganteum, Sorghum bicolor, Spinacia*

spp., Sporobolus fimbriatus, Stiburus alopecuroides, Stylosanthos humilis, Tadehagi spp, Taxodium distichum, Themeda triandra, Trifolium spp., Triticum spp., Tsuga heterophylla, Vaccinium spp., Vicia spp.Vitis vinifera, Watsonia pyramidata, Zantedeschia aethiopica, Zea mays, amaranth, artichoke, asparagus, broccoli, brussel sprout, cabbage, canola, carrot, cauliflower, celery, collard greens, flax, kale, lentil, oilseed rape, okra, onion, potato, rice, soybean, straw, sugarbeet, sugar cane, sunflower, tomato, squash tea, trees, grasses (including forage grass) and algae, amongst others.

According to a preferred feature of the present invention, the plant is a crop plant, such as soybean, sunflower, canola, rapeseed, cotton, alfalfa, tomato, potato, tobacco, papaya, squash, poplar, eucalyptus, pine, leguminosa, flax, lupinus and sorghum. According to a further preferred embodiment of the present invention, the plant is a monocotyledonous plant, such as sugarcane, further preferably the plant is a cereal, such as rice, maize (including forage corn), wheat, barley, millet, oats and rye.

Accordingly, the present invention provides any of the methods as described hereinabove, or a transgenic plant as described hereinabove, wherein the plant is a monocotyledonous crop plant, preferably a cereal, more preferably wherein the plant is rice or corn.

According to a particular embodiment of the invention, the plant is a dicotyledonous crop plant, or a dicotyledonous ornamental, such as azalea.

Advantageously, performance of the method according to the present invention leads to plants having a variety of improved growth characteristics relative to corresponding wild-type plants.

The term "growth characteristic" as used herein, preferably refers to, but is not limited to, increased yield/biomass or to any other growth characteristic as described hereinafter.

The term "yield" refers to the amount of produced biological material and is used interchangeably with "biomass". For crop plants, "yield" also means the amount of harvested material per acre or unit of production. Yield may be defined in terms of quantity or quality. The harvested material may vary from crop to crop, for example, it may be seeds (e.g. for rice, sorghum or corn when grown for seed); above-ground biomass (e.g. for corn, when used as silage), roots (e.g. for sugar beet, turnip, potato), fruits (e.g. for tomato, papaya), cotton fibers, or any other part of the plant which is of economic value. "Yield" also encompasses yield stability of the plants. High yield stability means that yield is not strongly affected by changes in environmental conditions, such as suboptimal conditions caused by drought, chilling, freezing, heat, salinity or nutrient deficiency. "Yield" also encompasses yield potential, which is the maximum obtainable yield under optimal growth conditions. Yield may be dependent on a number of yield components, which may be monitored by certain parameters. These parameters are well known to persons skilled in the art and vary from crop to crop. For example, breeders are well aware of the specific yield components and the corresponding parameters for the crop they are aiming to improve. For example, key yield parameters for corn include number of plants per hectare or acre, number of ears per plant, number of rows (of seeds) per ear, number of kernels per row, and thousand kernel weight. For silage corn, typical parameters are the above-ground biomass and energy content. Key yield parameters for rice include number of plants per hectare or acre, number of panicles per plant, number of flowers (spikelets) per panicle, seed filling rate (number of filled seeds per spikelet) and thousand kernel weight.

Generally, the term "increased yield" means an increase in biomass in one or more parts of a plant relative to the biomass of corresponding reference plants, for example relative to corresponding wild-type plants. The plants of the present invention exhibit increased plant size, manifested in taller plants and increased rosette diameter. Accordingly, the term "yield/biomass" as used herein encompasses increased plant size.

The plants of the present invention also exhibit increased organ size, and therefore, the term "increased yield/biomass" as used herein encompasses increased organ size. For example, the plants according to the present invention are characterized by increased size of the leaves, which is particularly important for forage and feed crops (and ornamentals). Furthermore, the plants exhibit increased size of the stem. Besides the contribution to increased yield, for example, in trees, an increase in stem thickness contributes to improved wind/rain resistance, for example in cereals. Furthermore, the plants according to the invention exhibit increased seed size.

The plants of the present invention exhibit an increased number of organs, and therefore, the term "increased yield/biomass" as used herein encompasses increased number of organs. For example, the plants according to the present invention exhibit an increased number of the leaves, which is particularly important for forage crops and ornamentals. Furthermore, the plants according to the present invention exhibit an increased number of the branches (lateral branches, rosette branches), which contributes to increased bushiness of the plant. Also, the plants according to the invention have increased number of trichome branches. An increased biomass of specialised epidermal outgrowth structures is advantageous in the production of cotton fibres or glandular trichomes. Specialised trichomes may also be used for the production of useful metabolites, pharmaceutical compounds, nutraceuticals and food additives. Furthermore, the plants according to the invention exhibit increased number of flowers, which is important for ornamentals and seed crops.

Also encompassed within the term "increased yield/biomass" is increased seed yield. Seed-yield may be manifested by increased total seed weight, increased number of total seeds, increased number of filled seeds, and/or increased seed size. An increase in seed size and/or volume may also influence the composition of seeds.

The term "growth characteristic" as used herein, also encompasses plant architecture. For example, the plants of the invention exhibit altered leaf shape, which may be advantageous for ornamental plant, and altered vascularization, which is important for wood and/or paper and pulp producing trees. The term "architecture" as used herein encompasses the appearance or morphology of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, number, position, texture, arrangement, and pattern of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, stem, petiole, trichome, flower, inflorescence (for monocots and dicots), panicles, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, sieve element, phloem or vascular tissue, amongst others. The term "architecture" therefore encompasses leaf area, leaf thickness, arrangement of lateral stems, stem shape and arrangement of flowers (and fruits).

The present invention also relates to use of a nucleic acid encoding a CCS52 protein or a variant thereof for improving plant growth characteristics, preferably for increasing yield, further preferably seed yield. Preferably, the nucleic acid is under the control of a medium-strength promoter.

Alternatively, increasing expression of a CCS52 nucleic acid, or introducing a CCS52 nucleic acid or the genetic construct into the plant cell, may be achieved by crossing or by breeding.

Furthermore, classical breeding techniques, aimed at improving plant growth characteristics, may be based on the selection of better performing allelic variants of a CCS52 gene, which better performing alleles may have an expression level that is higher than the wild-type level. Allelic variation may occur in nature, or may be created by mutagenic treatment of biological material, for example, by EMS mutagenesis. Therefore, the use of CCS52

allelic variants in breeding programmes, aimed at improving any of the growth characteristics as mentioned above, is also encompassed by the present invention; this may be in addition to their use in the methods according to the present invention. One example of a breeding program is a conventional marker-assisted breeding program.

Further information concerning the function of a CCS52 gene and related genes may be discovered by the use of reverse genetics, such a TILLING (Targeted Induced Local Lesions IN Genomes) in combination with the discovery of sites and motifs crucial for the gene and protein function (McCAllum et al., 2000, Plant Physiol 123(2):439-42; Perry et al., 2003 Plant Physiol 131(3):866-71). Plants having mutant or dominant negative, or dominant positive phenotypes may be analysed and compared to identify the most effective mutations. Phenotypes may be compared with phenotypes identified in, for example, QTL (Quantitative Trait Loci) analysis and sequence information may be compared with the gene mapping included in a QTL. Both methods may be useful when combined in identifying new phenotypes of interest for crop breeding.

The present invention will now be described with reference to the following figures in which:

Fig. 1 is a map of the entry clone, p1627, containing the gene of interest, CCS52A1, (CDS0198) within the AttL1 and AttL2 sites for Gateway® cloning in the pDONR201 backbone. This vector also contains a bacterial kanamycin-resistance cassette and a bacterial origin of replication.

Fig. 2 is a map of the binary vector for expression in *Arabidopsis thaliana* of the *Arabidopsis thaliana* CCS52A1 gene (CDS0198) under the control of a sunflower ubiquitin promoter (pUBIdeltaT). The CCS52A1 expression cassette further comprises the T-zein and T-rbcS-deltaGA double terminator sequence. This expression cassette is located within the left border

(LB repeat, LB Ti C58) and a right border (RB repeat, RB Ti C58) of the nopaline Ti plasmid. Cloned within these borders is also a selectable marker and a screenable marker, both under control of a constitutive promoter and followed by a nopaline (tNOS) or octopine (tOCS) transcription termination sequence. Furthermore, this vector also contains an origin of replication (pBR322 ori+bom) for bacterial replication and a bacterial selectable marker (Spe/SmeR) for bacterial selection.

Fig. 3 shows an aerial view of a wild-type *Arabidopsis thaliana* plant (left) and a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 transgene under control of an ubiquitin promoter (right). Both plants are 4 weeks old.

Fig. 4 shows a first cauline leaf of a wild-type *Arabidopsis thaliana* plant (left) and of a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under control of an ubiquitin promoter (right).

Fig. 5 shows a first rosette leaf of a wild-type *Arabidopsis thaliana* plant (left) and of a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under control of an ubiquitin promoter (right).

Fig. 6 shows leaf tissue of a wild-type *Arabidopsis thaliana* plant (left) and of a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under control of an ubiquitin promoter.

Fig. 7 shows epidermis and trichomes of a wild-type *Arabidopsis thaliana* plant (A) and of a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under control of an ubiquitin promoter (B).

Fig. 8 shows a wild-type *Arabidopsis thaliana* plant (left) and a transgenic *Arabidposis thaliana* plant expressing a CCS52A1 gene under the control of a 2S2 promoter (right), which are more bushier.

Fig. 9 shows a wild-type *Arabidopsis thaliana* plant (left) and a transgenic *Arabidposis thaliana* plant expressing a CCS52A1 gene under the control of an ubiquitin promoter (right).

Fig. 10 shows transversal sections of the main stem of a wild-type *Arabidopsis thaliana* plant (left) and of a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under control of an ubiquitin promoter (right).

Fig. 11 shows seeds produced by a wild-type *Arabidopsis thaliana* plant (left) and by a transgenic *Arabidopsis thaliana* plant expressing a CCS52A1 gene under the control of an ubiquitin promoter (right).

Fig. 12 shows a phylogentic tree of CCS52 related proteins in plants and animals. The sequences are presented by their Genbank accession number. Multiple sequence alignment across the entire sequences was done using CLUSTAL W (Higgins et al., (1994) Nucleic Acids Res. 22:4673-4680), with the BLOSSUM 62 matrix and with the parameters GAPOPEN 10, GAPEXT 0.05 and GAPDIST 8. The Phylogram view gives an estimate of phylogeny, i.e. branch lengths are proportional to evolutionary change.

Fig 13 shows the conserved consensus motifs in plant CCS52 related proteins.

Fig.14 shows the sequences of the present invention with their respective SEQ ID numbers.

Fig. 15 is a map of the binary vector p35S::AtCCS52A1 for expression in *Arabidopsis thaliana* of the *Arabidopsis thaliana* CCS52A1 gene (internal reference CDS0198) under control of the CaMV35S promoter. The CCS52A1 expression cassette further comprises a T-zein and T-rbcS-deltaGA double transcription termination sequence. This expression cassette is located within

the left border (LB repeat, LB Ti C58) and the right border (RB repeat, RB Ti C58) of the nopaline Ti plasmid. Within the T-DNA there is further provided a selectable and a screenable marker, both under control of a constitutive promoter and followed by a T-NOS or a T-OCS transcription terminator sequence. This vector further comprises an origin of replication (pBR322 ori + bom) for bacterial replication and a bacterial selectable marker (Spe/SmeR) for bacterial selection.

Fig 16. shows wild-type *Arabidopsis thaliana* plants and transgenic *Arabidopsis thaliana* plants transformed with the vector carrying the p35S::AtCCS52A1 expression cassette.

Fig. 17 is a map of the binary vector pEXP::AtCCS52A1 for expression in *Oryza sativa* of the *Arabidopsis thaliana* CCS52A1 gene (internal reference CDS0198) under the control of the rice beta-expansin promoter. The CCS52A1 expression cassette further comprises a T-zein and T-rbcS-deltaGA double transcription termination sequence. This expression cassette is located within the left border (LB repeat, LB Ti C58) and the right border (RB repeat, RB Ti C58) of the nopaline Ti plasmid. Within the T-DNA there is further provided a selectable and a screenable marker, both under control of a constitutive promoter and followed by polyA or a T-NOS transcription terminator sequence. This vector further comprises an origin of replication (pBR322 ori + bom) for bacterial replication and a bacterial selectable marker (Spe/SmeR) for bacterial selection.

EXAMPLES

The present invention will now be described with reference to the following examples, which are by way of illustration alone.

DNA Manipulation

Unless otherwise stated, recombinant DNA techniques are performed according to standard protocols described in Sambrook (2001) Molecular

Cloning: a laboratory manual, 3rd Edition Cold Spring Harbor Laboratory Press, CSH, New York or in Volumes 1 and 2 of Ausubel et al. (1998), Current Protocols in Molecular Biology. Standard materials and methods for plant molecular work are described in Plant Molecular Biology Labfase (1993) by R.D.D. Croy, published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications (UK).

Example 1: ***Cloning of Arabidopsis thaliana CCS52A1***

The *Arabidopsis* CCS52A1 gene (internal reference CDS0198) was amplified by PCR using as template an *Arabidopsis thaliana* seedling cDNA library (Invitrogen, Paisley, UK). After reverse transcription of RNA extracted from seedlings, the cDNA fragments were cloned into pCMV Sport 6.0. Average insert size of the cDNA library was 1.5 kb, and original number of clones was about 1.59×10^7 cfu. The original titer of 9.6×10^5 cfu/ml was brought to 6×10^{11} cfu/ml after amplification of the library. After plasmid extraction of the clones, 200 ng of plasmid template was used in a 50 µl PCR mix. The primers used for PCR amplification, prm01391 with the sequence 5' *GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACA*ATGGAAGAAGAAGATC CTACAGC 3' (SEQ ID NO 18) and prm01392 with the sequence 5' *GGGGACCACTTTGTACAAGAAAGCTGGGT*TTCTCACCGAATTGTTGTTCTA C 3' (SEQ ID NO 19) an AttB site for Gateway recombination cloning (italics). PCR was performed using Hifi Taq DNA polymerase in standard conditions. A PCR fragment of the expected length was amplified and purified also using standard methods. The first step of the Gateway procedure, the BP reaction, was then performed, during which the PCR fragment recombines *in vivo* with the pDONR201 plasmid to produce the "entry clone", p1627 (Fig. 1). Plasmid pDONR201 was purchased from Invitrogen, as part of the Gateway® technology.

Example 2: ***Vector construction (pUBI::AtCCS52A1)***

The entry clone p1627 was subsequently used in an LR reaction with p0712, a destination vector used for *Arabidopsis thaliana* transformation. This vector contains as functional elements within the T-DNA borders, a plant selectable marker, a screenable marker and a Gateway cassette intended for LR *in vivo* recombination with the sequence of interest already cloned in the entry clone. Upstream of this Gateway cassette lies the sunflower ubiquitin promoter (internal reference PRO155) for constitutive expression of the gene of interest. After the LR recombination step, the resulting expression vector pUBI::AtCCS52A1 (Fig. 2) was transformed into *Agrobacterium* strain LBA4044 and subsequently into *Arabidopsis thaliana* plants as described in Example 3.

Example 3: ***Arabidopsis transformation***

Sowing and growing of parental plants

For the parental plants, approximately 12 mg of wild-type seeds from *Arabidopsis thaliana* (ecotype Columbia) was suspended in 27.5 ml of 0.2 % agar solution. The seeds were incubated for 2 to 3 days at a temperature of 4°C and were then sown. The seeds were then allowed to germinate under the following standard conditions: 22°C during the day, 18°C at night, 65-70% relative humidity, 12 hours of photoperiod, sub-irrigation with water for 15 min every 2 to 3 days. The developed seedlings were planted in pots of 5.5 cm diameter, containing a mixture of sand and peat (ratio 1:3). The plants were allowed to grow under the same standard conditions as mentioned above.

Agrobacterium growth conditions and preparation

Agrobacterium strain C58C1RIF with helper plasmid pMP90 containing vector pUBI::AtCCS52A1 was inoculated in a 50 ml plastic tube containing 1 ml Luria Broth (LB) without antibiotics. The culture was shaken at 28°C for 8-9h. After addition of 10 ml of LB without antibiotic, the plastic tube was shaken overnight at 28°C. The OD at 600 nm was monitored. At an optical density of

approximately 2.0, 40 ml of 10% sucrose and 0.05% Silwet L-77 (a chemical mixture of polyalkyleneoxide modified heptamethyltrisiloxane (84%) and allyloxypolyethyleneglycol methyl ether (16%), OSI Specialties Inc.) was added to the culture. The *Agrobacterium* culture obtained was labelled CD2175 and used to transform the parental *Arabidopsis* plants.

Flower dip

When each parental plant had one inflorescence of 7-10 cm in height, the inflorescences were inverted into the *Agrobacterium* culture and agitated gently for 2-3 seconds. 2 plants per transformation were used. Subsequently, the plants were returned to normal growing conditions as described above.

Seed collection

5 weeks after the flowers were dipped in the *Agrobacterium* culture, watering of the plants was stopped. The plants were incubated at 25°C with a photoperiod of 20 hours. One week later, the seeds were harvested and placed in a seed drier for one week. The seeds were then cleaned and collected in 15 ml plastic tubes. The seeds were stored at 4°C until further processing.

Example 4: *Evaluation of transformed Arabidopsis plants*

Selection of the first generation of transgenic plants

100 mg of seeds were placed in a 50 ml plastic tube and suspended in 27 ml of a 0.2% agar solution. The tubes were stored at 4° C for 3 days to release the seeds from dormancy. Following this period, the seed suspension was examined under blue light to determine the presence of transformed seeds. 20 bright fluorescent seeds (expressing the selectable marker) were aspirated with a Pasteur pipette, transferred to a 15ml plastic tube, and the suspension volume was adjusted to 15ml with a 0.2% agar solution. The same amount of non-fluorescent seed was transferred to a separate 15ml plastic tube and the suspension volume adjusted to 15ml with a 0.2% agar solution. The suspension of expressing seeds was evenly dispensed as drops of 50 µl on

one half of a 50x30cm tray containing a mixture of sand and soil in a ratio of 1 to 2. The non-expressing seeds were dispensed in the same way on the other half of the tray. The tray was placed in a greenhouse under the following conditions: 22°C during the day, 18°C at night, 60% relative humidity, 20 hour photoperiod, sub-irrigation once a day with water for 15 min. On the 14th day after sowing, 5 expressing and 5 non-expressing seedlings were transplanted into individual pots of 10 cm diameter filled with a mixture of sand and peat (ratio 1:3).

Cultivation and imaging of the first generation of transgenic plants

The pots were then placed in a greenhouse under the same conditions as described for the trays. The pots were sub-irrigated for 15 minutes, once a week, or more if needed. On the 21st, 28th, 35th, 42nd and 49th day after sowing, the rosettes of each plant were photographed using a digital camera. On the 35th, 42nd, 49th and 56th day after sowing, the inflorescence of each plant was photographed, using a digital camera. The number of pixels corresponding to plant tissues was recorded on each picture, converted to cm^2 and used as a measurement of plant size. On the 57th day after sowing, when the first siliques were ripening, a breathable plastic bag was placed on each plant and tightly attached at the base of the plants to collect the shedding seeds. On the 90th day after sowing, when all the siliques were ripe, the seeds were collected and placed in a seed drier for 1 week before storage in a sealed container at 4°C.

Seed yield of the first generation of transgenic plants

Harvested inflorescences of the T1 plants were taken and gently rubbed to release seeds from the siliques. The mixture of seeds and chaff was then passed over a mesh to remove large fragments of stems, leaves, siliques, etc. The seeds were then poured onto a vibrating gutter equipped with a vacuum cleaner allowing the lighter fragments, such as petals and small fibers, to be aspirated whilst retaining the heavier seeds. Data on the seed parameters were measured using an automated system.

A similar procedure was followed to evaluate the phenotypic characteristics of *Arabidopsis* T2 lines. At least 15 expressing and at least 15 non-expressing seedlings were transplanted into individual pots with a diameter of 10 cm (containing a mixture of sand and peat in a ratio of 1 to 3) and processed as described above. The phenotypic characteristics, as described above, were inherited to further generations.

Example 5: ***Phenotypic characteristics of pUBI::AtCCS52A1 transgenic plants***

Increased biomass

CCS52 transgenic plants showed increased biomass relative to control plants. This was manifested by increased leaf size (see Fig. 4, 5 and 6).

Increased leaf biomass was also manifested by increased number of rosette leaves (see Fig. 3) and increased number of cauline leaves (Fig. 8).

Increased biomass was further manifested by increased stem thickness and more branching, which leads to a bushy phenotype. As illustrated in Fig. 9 and Fig. 10, pUBI::CCS52 transgenic plants have an increased rosette diameter as well as an increased (main) stem diameter and an increased diameter of the lateral branches. As a consequence it is estimated that overall plant biomass is multiplied by 3 to 4 in CCS52 transgenic *Arabidopsis* plants.

Modified trichomes

As shown in Fig. 7, transgenic plants have trichomes with increased number of branches relative to the wild-type trichomes.

Modified plant and organ shape

As shown in Fig. 4, the cauline leaf of the transgenic plant was of a different shape and of a larger size than the corresponding wild-type plant. As shown in Fig. 5, the rosette leaf of the transgenic plant had increased width and a larger area than the corresponding wild-type leaf. Further, this figure

illustrates that a substantial increase of the vascularisation system was visible in the transgenic leaf.

Increase yield - seed yield

As shown in Fig. 11, seed size was enlarged in the pUBI::CCS52 transgenic plant.

Example 6: ***Overexpression of AtCCS52A1 under control of the 2S2 promoter resulted in bushier plants***

Starting from the entry clone p1627, an expression vector was made in a similar way as described in Examples 1 and 2, except that the promoter upstream of the AtCCS52A1 gene was the *Arabidopsis* 2S2 seed-preferred promoter. This expression vector was transformed into *Arabidopsis* as described in Example 3 and plant evaluation was carried out as described in Example 4.

The phenotypic characteristics of the p2S2::CCS52 transformed plants was similar as the pUBI::CCS52 transformed plants described in Example 5. It was observed that p2S2::CCS52 transformed plants had increased biomass of leaves, increased number of branches and/or increased biomass of stems. As further illustrated in Fig. 8, the p2S2::CCS52 transgenic plant had an increased number of leaves, at least 2 times more rosette branches, thicker stems and more lateral branches, which gave rise to a bushier phenotype. Furthermore, these plants showed more flowers.

Example 7: ***Overexpression of CCS52 under control of the CaMV35S promoter in Arabidopsis resulted in small, aberrant plants***

Starting from the entry clone p1627, an expression vector was made in a similar way as described in Examples 1 and 2, except that the promoter upstream of the AtCCS52A1 gene was the CaMV35S promoter. The resulting

expression vector p35S::AtCCS52A1 (Fig. 15) was transformed into *Arabidopsis* as described in Example 3 and plant evaluation was carried out as described in Example 4.

Arabidopsis plants were regenerated and grown under optimal growth conditions as mentioned in Example 4. Nullizygote plant without the transgene were alternated with transgenic plant comprising the transgene in a growing tray (Fig. 16). During growth in optimal conditions, a significant difference between transgenic and wild-type plant was observed. After 5 to 6 weeks the plants were photographed (Fig. 16). At this stage the transgenic plants showed a small and aberrant phenotype compared with the mature and healthy wild-type plant. The transgenic plant clearly had smaller leaves, smaller or no stems, smaller rosette diameter, fewer leaves and fewer flowers compared to the wild-type plant. Clearly the p35S::CCS52 transgenic plants suffered from an early growth arrest. These transgenic plants are small and have aberrant organ formation. In transgenic plants the leaves were reddish, indicating that these plant suffered from stress and the aberrant plants produced significantly reduced amounts of siliques and seeds, compared to wild-type plants.

Example 8: ***Overexpression of AtCCS52 under control of different medium-strength promoters in rice***

Starting from the entry clone p1627, different expression vectors are made in a similar way as described in Examples 1 and 2, except that the destination vector for the LR recombination reaction is a destination vector useful for transformation of *Oryza sativa*. This destination vector carries as functional elements within the T-DNA borders, a plant selectable marker, a screenable marker and a Gateway cassette intended for LR *in vivo* recombination with the CCS52 sequence already cloned in the entry clone. Different versions of this destination vector have different medium-strength promoters upstream of this Gateway cassette. The different resulting

expression vectors therefore have different promoters upstream of the CCS52 gene.

One example of such an expression vector, pEXP::AtCCS52A1 carrying the rice beta-expansin promoter (PRO0061) upstream of the AtCCS52A1 gene, is represented in Fig. 17. Other examples of expression vectors are CD02376, carrying the rice prolamin promoter (PRO090); or CD05509, carrying the rice Oleosin 18kDa promoter (PRO0218); or CD13390, carrying the rice putative protochlorophyllide reductase promoter (PRO0123), or a vector carrying the methallothionein promoter upstream of the AtCCS52A1 gene.

Similar vectors are made, for the expression of other CCS52A genes or CCS52B genes under control of the promoters as mentioned hereinabove.

All these expression vectors are suitable for the transformation of rice following the protocols as mentioned hereinabove.

*At*CCS52A1 transgenic rice plants, overexpressing AtCCS52A1 under control of a medium-strength promoter, have improved growth characteristics. Especially, the transgenic rice plants have increased yield/biomass, manifested by increased plant size (increased plant area and/or increased plant height) or increased harvest index, which is the ratio of the total biomass over the harvested biomass. Increased biomass is also manifested by increased organ size such as increased leaf size, increased seed size (increased thousand kernel weight (TKW)), increased seed yield/seed biomass or increased stem diameter. Increased biomass is also manifested by increased number of organs such as increased number of leaves, increased number of branches, increased number of tillers, increased number of panicles, increased number of flowers, increased number of seeds or increased number of filled seeds or increased filling rate. Further these transgenic rice plants show early flowering (shorter life cycle), compared to the corresponding nullizygotes.

Example 9: ***Overexpression of CCS52 under control of a medium-strength promoter in corn***

Similar constructs as described in Example 7 are made for the transformation of corn and the methods of the invention described herein are also used in corn (*Zea mays*). To this aim, a CCS52 gene, for example, a corn orthologue, is cloned under control of a promoter operable in corn, in a plant transformation vector suitable for *Agrobacterium*-mediated corn transformation. The promoter operable in corn may for example, be a medium-strength promoter, which is constitutive, for example, an ubiquitin promoter or any of the useful promoters as mentioned hereinabove. Methods to use for corn transformation have been described in literature (Ishida et al., Nat Biotechnol. 1996 Jun;14(6):745-50; Frame et al., Plant Physiol. 2002 May;129(1):13-22).

Transgenic (inbred) lines made by these methods may be crossed with another non-transgenic or transgenic (inbred) line or be self/sib-pollinated. Importantly, transgenic (inbred) lines may be used as a female or male parent. Inheritability and copy number of the transgene are checked by quantitative real-time PCR and Southern blot analysis and expression levels of the transgene are determined by reverse PCR and Northern analysis. Transgenic events with single copy insertions of the transgene and with varying levels of transgene expression are selected for further evaluations in subsequent generations.

Progeny seeds obtained as described hereinabove are germinated and grown in the greenhouse in conditions well adapted for corn (16:8 photoperiod, 26-28°C daytime temperature and 20-24°C night time temperature) as well under water-deficient, nitrogen-deficient, and excess NaCl conditions. Null segregants from the same parental line (inbred line or hybrids), as well as wild-type plants of the same inbred line or hybrids are used as controls. The progeny plants are evaluated on different biomass and developmental parameters, including but not limited to plant height, stalk width, nodes below ear, nodes above ear, brace roots, number of leaves, leaf greenness, leaf

angle, total above-ground area time to tassel, time to silk, time to maturity, ear height, ear number, ear length, ear weight, row number, kernel number, grain moisture. Kernel traits include but are not limited to kernel size, kernel weight, starch content, protein content, and oil content are also monitored. Corn yield is calculated according to well-known methods. Corn plants transformed with a CCS52 protein show improved growth characteristics. More particularly they show an improvement in any one or more of the abovementioned biomass and developmental parameters.

Transgenic events that are most significantly improved compared to corresponding control lines are selected for further field-testing and marker-assisted breeding, with the objective of transferring the field-validated transgenic traits into another germplasm. The phenotyping of maize for growth and yield-related parameters in the field is conducted using well-established protocols. The corn plants are particularly evaluated on yield components at different plant densities and under different environmental conditions. Subsequent improvements for introgressing specific loci (such as transgene containing loci) from one germplasm into another is also conducted using well-established protocols including but not limited to MAS.

<u>Example 10</u>: Overexpression of AtCCS52A2, AtCCS52B or orthologues from other plants, such as OsCCS52A

The experiments as described in Examples 7 to 9 are repeated with other CCS52 genes.

The AtCCS52A2 (internal reference CDS0199) is cloned under control of the rice Oleosin 18kDa promoter (PRO0128) in vector CD04769, the rice Prolamin promoter (PRO0090) in vector CD04778, the rice beta-expansin promoter (PRO0061) in vector CD13386 or the rice putative protochlorophyllide reductase promoter (PRO0123) in vector CD13522.

AtCCS52B (CDS0390) is cloned under control of the rice prolamin promoter (PRO0090) in the vector CD02164, the rice beta-expansin promoter (PRO0061) in vector CD13388 or the rice metallothionein promoter (PRO0126) in the vector CD13530.

Plants transformed with a CCS52 gene under the control of a medium-strength promoter, for example, transformed with one of the constructs as mentioned above, show improved growth characteristics, such as increased plant size, increased organ size and/or increased number of organs.